EXCELLENT PUBLISHERS

International Journal of Current Research and Academic Review

ISSN: 2347-3215 Volume 3 Number 12 (December-2015) pp. 70-76

www.ijcrar.com

Molecular Identification of *Aspergillus flavus* Strain PKP2 Involved in Synthesis of Silver Nanoparticles

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KEYWORDS

Aspergillus flavus, Silver nanoparticles, 18S rDNA sequencing

A B S T R A C T

In this study, the extracellular production of silver nanoparticles by the fungus *Aspergillus flavus* was investigated. The molecular identification of a selected fungal isolate was performed by partial 18S rDNA sequencing. This fungal isolate PKP2 was found to be closely related to Aspergillus nomius strain SGE19 with 99% sequence similarity. The 702 bp 18S rDNA nucleotide sequence was provided a GenBank accession number KT369092. The study emphasizes that Sirumalai hill soil can support the growth of nanoparticle synthesizing fungi and subsequent production of silver nanoparticles.

Introduction

The biosynthesis of nanoparticles has received increasing attention due to the growing need to develop safe, cost effective and environment friendly technologies for nanoparticles synthesis. Out of all kinds of nanoparticles, the metallic nanoparticles, including gold, silver iron, zinc and metal oxide nanoparticles, have shown great promise in terms of biomedical applications, not only due to their large surface area to volume ratio but also because they exhibit different biomedical applications. Biotechnological approach towards the synthesis of nanoparticles has many advantages such as economic viability, possibility of easily covering large surface area by suitable growth of the mycelia, and its green chemistry nature.

In the present study, we have isolated, screened fungal strain for biosynthesis of silver nanoparticles and the synthesized AgNPs were characterized by UV-Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FT-IR), X-Ray diffractometer (XRD) and Scanning Electron Microscope (SEM) analysis. The application of molecular techniques such as PCR based identification is being increasingly used for

microbial species identification as it is rapid, specific and sensitive.

Materials and Methods

The fungus *Aspergillus fumigatus* was isolated from the soil samples of Sirumalai hills, Dindigul district, TamilNadu. The fungal culture was maintained in Potato Dextrose Agar slants at 25° C. The fungus was grown in 500 mL Erlenmeyer flasks, each containing 100 mL MGYP media composed of malt extract (0.3%), glucose (1.0%), yeast extract (0.3%), and peptone (0.5%) at 25-28 °C under shaking conditions (200 rpm) for 96 h. After 96 h of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 10 °C for 20 min, and the settled mycelia was washed thrice with sterile distilled water. Ten grams of the harvested mycelial mass was then resuspended in 100 mL sterile distilled water in 500 mL conical flasks. To this suspension, 100 mL of an aqueous solution of 2×10^{-4} M $AgNO_3$ was added.

The reaction was carried out in dark. The control without silver ion was run along with the test sample. After an incubation of about 72 hours, the filtrate containing silver nanoparticles were characterized by using UV- visible spectrophotometer (Bhainsa and D'Souza, 2006), Fourier Transform Infrared spectroscopy, X-Ray diffraction analysis (Mukherjee *et al*., 2001) and SEM analysis (Vigneshwaran *et al*., 2007; Talebia *et al*., 2010). Also changing of solution colour from pale yellow to dark brown was confirmed, thus silver nanoparticles was used further to continuing the experiment.

Molecular Identification

The genomic DNA isolation, PCR amplification and partial 18S rDNA sequencing of the aflatoxigenic fungal isolate were performed.

Extraction of Fungal Genomic DNA

Pure culture of the fungus was inoculated from 5 days old PDA plate into 20 ml of potato dextrose broth and incubated at 25°C and 150 rpm. Mycelia from 2 days old culture were harvested by filtration through Whatman No.1 filter paper and used for genomic DNA isolation using Fungal Genomic DNA Spin-50 isolation kit according to the manufacturer's instructions. The eluted DNA was used for PCR amplification.

PCR Amplification

DNA amplification by polymerase chain reaction (PCR) was performed in a total volume of 100 µl. Each reaction mixture contained the following solutions: 1 µl template DNA, 400 ng forward universal 18S rDNA primer NS1 (5'-GTAGTCATATGCTTGTCTC-3'); 400 ng reverse 18S rDNA primer C18L (5'-GAAACCTTGTTACGACTT-3'); 4 µl of dNTPs (2.5 mM each); 10 µl of Taq DNA polymerase assay buffer and 1 µl Taq DNA polymerase (3 U/µl) and water was added up to 100 µl.

The ABI 2720 Thermal Cycler (Applied Biosystems, USA) was programmed as follows: 5 min initial denaturation at 94°C, followed by 35 cycles that consisted of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension at 72°C for 1 min and a final extension of 5 min at 72°C. PCR products obtained were eluted from the gel using Gel Extraction Spin-50 kit according to the manufacturer's instructions. The PCR amplified product was detected by 1.2% agarose gel (with ethidium bromide) electrophoresis.

Partial 18S rDNA Sequencing and Analysis of Sequence Data

Sequencing of the PCR amplified product was performed using BigDye Terminator Version 3.1 Cycles Sequencing kit and ABI 3500xL Genetic Analyzer (Applied Biosystems, USA). 10 µl of the sequencing reaction mixture contained 4 µl of BigDye Terminator Ready Reaction Mix, 1 µl of rDNA amplification product (100 ng/µl), 2 µl primer (10 pmol/λ) and 3 µl Milli-Q Water. The ABI 2720 Thermal Cycler (Applied Biosystems, USA) was programmed to perform initial denaturation at 96°C for 1 min, followed by 25 cycles that consisted of denaturation at 96°C for 10 s, hybridization at 50°C for 5 s and elongation at 60°C for 4 min. The resultant nucleotide sequence was analyzed using the software Seq Scape version 5.2. The fungal species was identified by comparing the sequence with known 18S ribosomal sequences in the NCBI database using BLASTN. The phylogenetic tree was constructed based on fast minimum evolution method using BLAST pairwise alignment between the query and the database sequences. The nucleotide sequence was submitted to GenBank database (NCBI, USA) under an accession number.

Results and Discussion

The biomass that mixing with the aqueous solution of Ag ions, the color of the biomass changed from pale yellow to brownish (Fig.1) after 72 hrs of incubation that was the first symptom of nanosilver production. UV-Visible spectrophotometer results showed a peak at 430nm (Fig. 2). The lyophilized nanoparticle samples were analyzed in FTIR. The representative spectra of Silver nanoparticles were showed (Fig. 3). Functional Groups corresponding to the absorption peaks were enumerated (Table.1). X-RD pattern was compared with standard pattern and evidenced for production of nanosilver crystals (Fig. 4). Furthermore SEM images of silver Nanoparticles showed the size range 7 nm (Fig. 5).

Molecular Identification of Fungal Isolate PKP2

The molecular identification of the fungal isolate PKP2 was performed by partial 18S rDNA sequencing. The apparent size of the PCR amplicon was 1.5-1.8 kb, shown in Fig. 6.

Table.1 FTIR Functional Groups Analyses

Wave number (cm^{-1})	Bond	Functional group
3434.74	O-H stretch, H-bonded	Alcohols, Phenols
2077.57	-NCS	Isothiocyanate
1637.08	N-H bend	Primary amines
1444.40	C-C Stretch (in ring)	Aromatics
1350.91	C-H rock	Alkanes
681.57	C-Br Stretch	Alkyl halides

Fig.1 Change of Colour from Pale Yellow (Left) to Brown Colour (Right)

Fig.2 UV Analysis of AgNP

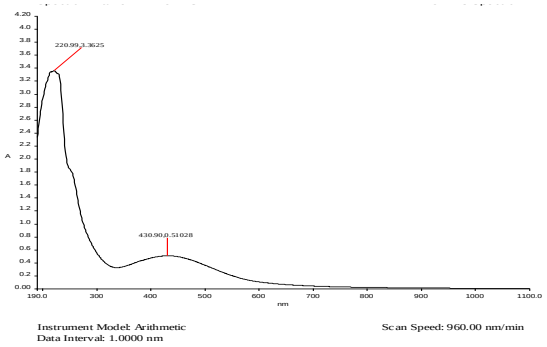

Fig.3 FT-IR Analysis of Biomolecules Involved in Reduction AgNO3 into Ag ions

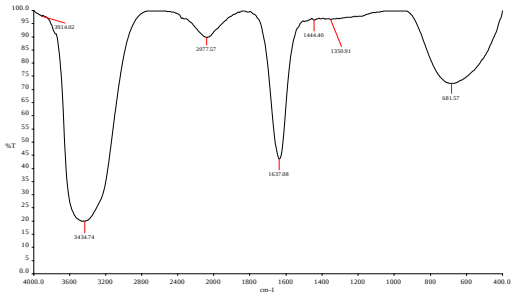

Fig.4 XRD Showing Peak Indices

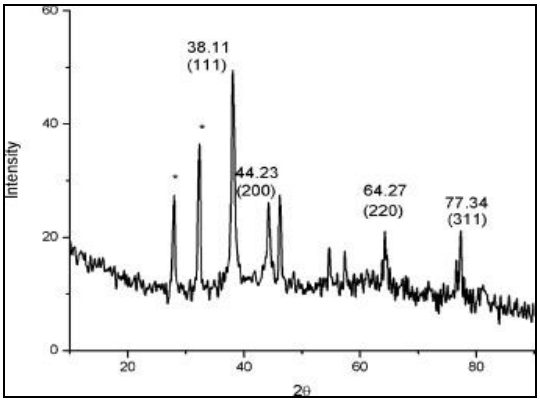

Fig.5 SEM picture of Silver Nanoparticles

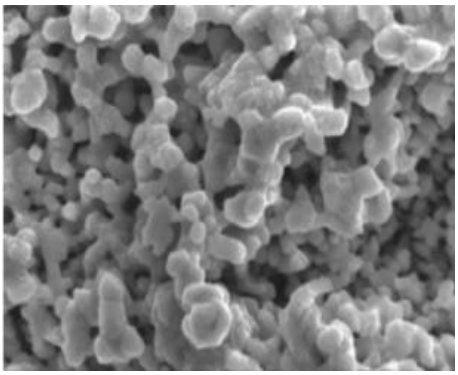

Fig.6 Agarose gel Analysis of PCR Amplification Product using Universal 18S rDNA Primers. Lane M, 500 bp DNA Molecular Size Marker; Lane 1, Fungal Isolate PKP2

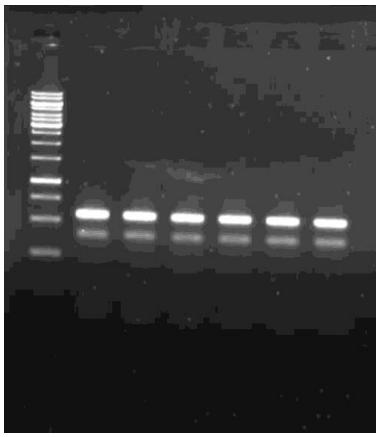

Fig.7. The 702 bp 18S rDNA Nucleotide Sequence of Fungal Isolate PKP2

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   1 ggggctacgg agtgtaggtt cctagcgagc ccaacctccc acccgtgttt actgtacctt
  61 agttgcttcg gcgggcccgc cattcatggc cgccgggggc tctcagcccc gggcccgcgc
 121 ccgccggaga caccacgaac tctgtctgat ctagtgaagt ctgagttgat tgtatcgcaa
 181 tcagttaaaa ctttcaacaa tggatctctt ggttccggca tcgatgaaga acgcagcgaa
 241 atgcgataac tagtgtgaat tgcagaattc cgtgaatcat cgagtctttg aacgcacatt
 301 gcgccccctg gtattccggg gggcatgcct gtccgaacgt cattgctgcc catcaagcac
 361 ggcttgtgtg ttgggtcgac gtcccctctc ctgggggggac ttttccccaa ggcagcgtcg
 421 gtaccgtttg cgatcctcga gcataaggct gttttgccac ccgctctgta ggccctgcct
 481 gcgcttgccg aaggaaaatc attcttttcc cagggttgac ctcggatcag ggaaggaata
 541 ccgtcggaat taatctttcc ataaccgtga aaggaaactt ttactaatgg tttggtttct
 601 aagccgccac tctccacgcg tgtttagtgt accttagttg cctctgcggg cccgaccttc
 661 aaggtcgccg ggggctctca gcccgggccg cgccggcgga aacccacgat atctatctga
 721 tctatcgtac tctgaattgt ttgccatcaa catcattaaa tatctcacaa ggcatcttcg
 781 gttactcgtt caattactac gcgcaataat gccatacata ctgtgactgt gcaatactct
 841 ccacccccact catttcagca ctagcgtccg cgcactcagg gggggaggag gacacacttt
 901 tgagctaaca cacgattcgt ggcctgtgca tta
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Fig.8 Phylogenetic Tree Showing the Relationship between the Fungal Isolate PKP2 (unknown) and Related Ascomycetes from NCBI Database Based upon Fast Minimum Evolution Analysis of partial 18S rDNA Sequences

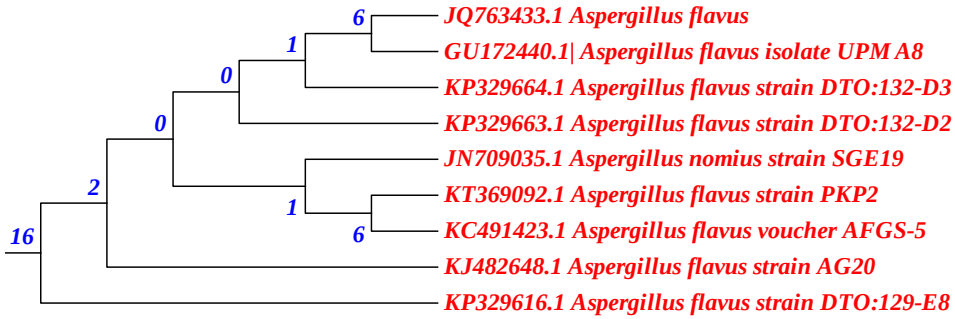


Fig.9 Secondary Structure of the Fungal Isolate PKP2

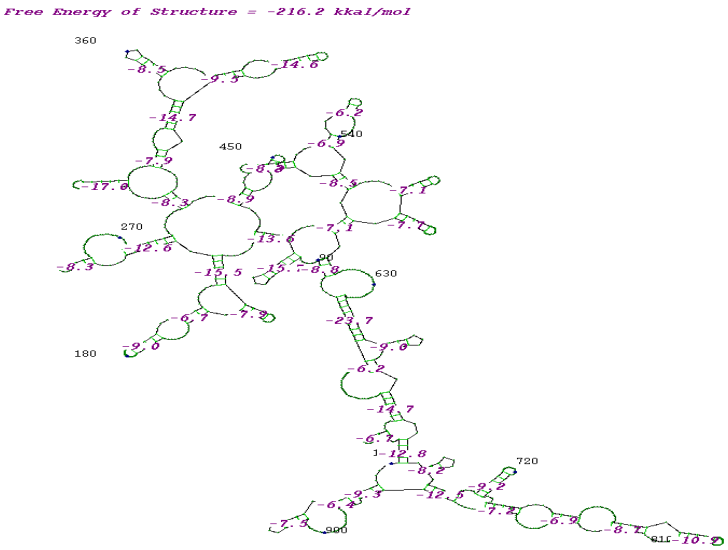
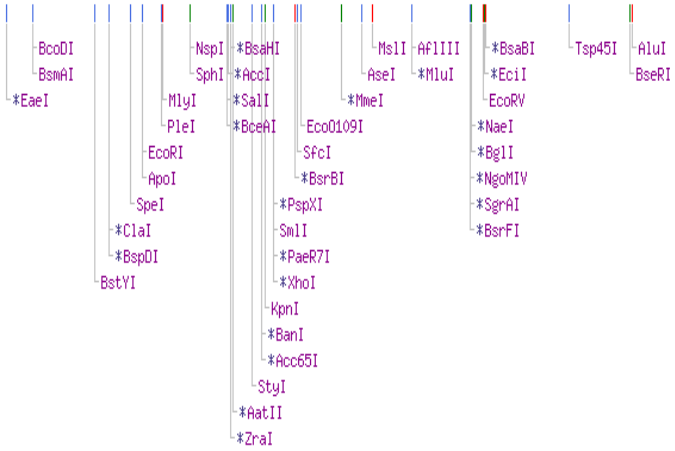


Fig.10 Restriction Sites of the Fungal Isolate PKP2



Various molecular approaches that target the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region and the internal transcribed spacer (ITS) regions have been used previously for rapid detection of *Aspergillus* from environmental and clinical samples (Einsele *et al*., 1997). In a recent study species of *Aspergillus* were identified by comparing partial 18S rDNA sequences from 57 different fungal isolates with the available ribosomal sequences using BLAST search (Oktay *et al*., 2011). Automated molecular techniques that would combine extraction of microbial DNA from clinical materials, DNA amplification and amplicon detection are currently under commercial development for identification of fungal pathogens (Loeffler *et al*., 2002).

Conclusion

It can be concluded that *Aspergillus flavus* may be commonly isolated from Sirumalai hill soil. The identity and phylogenetic relationship of such a fungal isolate can be established by 18S rDNA sequencing. The present study also emphasizes that Sirumalai hill soil can support the growth of fungal isolate PKP2 and subsequent production of silver nanoparticles.

Acknowledgement

The authors sincerely acknowledge the Secretary and Correspondent, Principal, A.V.V.M. Sri Pushpam College, Poondi for their support in all the way. They also thank Mr.A.S.Vijayakumar, Director, Carl Neuberg Research Laboratory, Pudukkottai for providing laboratory facilities.

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